

Application No. : 09/709,785  
Docket No. : 660088.433C1

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification:

Paragraph beginning at page 15, line 19, has been amended as follows:

Figure 1 shows the nucleotide sequences of the coding regions of human ANT1 ("ANT1m"), (SEQ ID NO: 1), human ANT2 ("ANT2m") (SEQ ID NO: 57) and human ANT3 ("ANT3m") (SEQ ID NO: 3).

Paragraph beginning at page 15, line 21, has been amended as follows:

Figure 2 shows the polypeptide sequences of human ANT1 ("ANT1p") (SEQ ID NO: 47), human ANT2 ("ANT2p") (SEQ ID NO: 48) and human ANT3 ("ANT3p") (SEQ ID NO: 49).

Paragraph beginning at page 18, line 27, has been amended as follows:

ANT and CypD polypeptides as provided herein may comprise one or more deduced amino acid sequences of Figures 1 and 8 [~~SEQ ID NOS: \_\_\_ and \_\_\_~~] (SEQ ID NOS: 1, 57, 3 and 39). Alternatively, such polypeptides may comprise one or more polypeptide sequences encoded by ANT/CypD nucleic acid expression constructs as provided herein. Polypeptides comprising fragments, analogs and derivatives of such polypeptide sequences, as well as fusion proteins, are further contemplated. As used herein, the terms "fragment," "derivative" and "analog" when referring to ANT and CypD polypeptides or fusion proteins, refer to any ANT and CypD polypeptides or fusion proteins that retain essentially the same biological function or activity as such polypeptide. For example, an analog may be a proprotein that can be activated by cleavage to

produce an active ANT and/or CypD polypeptide. The polypeptide of the present invention may be a recombinant polypeptide or a synthetic polypeptide, and is preferably a recombinant polypeptide.

Paragraph beginning at page 21, line 28, has been amended as follows:

The polypeptides of the present invention include ANT and CypD polypeptides and fusion proteins having amino acid sequences that are identical or similar to sequences known in the art. For example, by way of illustration and not limitation, the human ANT and CypD polypeptides of Figures 1, 2, 7 and 8 [~~SEQ ID NOS: \_\_\_\_\_~~] (SEQ ID NOS: 47-49, 27 and 40) are contemplated for use according to the instant invention, as are polypeptides having at least 70% similarity (preferably a 70% identity) to the polypeptides of Figures 1, 2, 7 and 8 and more preferably 90% similarity (more preferably a 90% identity) to the polypeptides of Figures 1, 2, 7 and 8 and still more preferably a 95% similarity (still more preferably a 95% identity) to the polypeptides of Figures 1, 2, 7 and 8 and to portions of such polypeptides, wherein such portions of ANT and CypD polypeptides generally contain at least 8 consecutive amino acids, preferably at least 12 consecutive amino acids, more preferably at least 20 consecutive amino acids, more preferably at least 30 consecutive amino acids and more preferably at least 50 consecutive amino acids.

Paragraph beginning at page 28, line 10, has been amended as follows:

As described herein, the invention provides ANT and CypD fusion proteins comprising ANT or CypD polypeptides fused to an additional functional or non-functional polypeptide sequence that permits, for example by way of illustration and not limitation, detection, isolation and/or purification of the ANT and CypD fusion proteins. For instance, an additional functional polypeptide sequence may be an energy transfer molecule polypeptide as provided herein. ANT and CypD fusion proteins described herein may be detected by FRET, fluorescence, phosphorescence, bioluminescence, or chemiluminescence, and include fusion proteins that may in certain embodiments be detected, isolated and/or purified by protein-protein affinity (*e.g.*, receptor-ligand), metal affinity or charge affinity methods. In certain other embodiments the subject invention fusion proteins may be detected by specific protease cleavage of a fusion protein having a sequence that comprises a protease recognition sequence, such that the ANT and CypD

polypeptides may be separable from the additional polypeptide sequence. In particularly preferred embodiments, for example, each ANT and/or CypD polypeptide sequence is fused in-frame to an energy transfer molecule polypeptide sequence. Other polypeptide sequences present in ANT and CypD fusion proteins may facilitate affinity detection and isolation of ANT and CypD polypeptides and may include, for example, poly-His or the defined antigenic peptide epitopes described in U.S. Patent No. 5,011,912 and in Hopp et al., (1988 *Bio/Technology* 6:1204) (e.g., FLAG<sup>®</sup> epitope tag DYKDDDDK, {~~SEQ ID NO: 54~~} (SEQ ID NO: 55), or the XPRESS<sup>™</sup> epitope tag {~~SEQ ID NO: 55~~} (DLYDDDDK, SEQ ID NO: 56; Invitrogen, Carlsbad, CA). The affinity sequence may be a hexahistidine tag as supplied, for example, by a pBAD/His (Invitrogen) or a pQE-9 vector to provide for purification of the mature polypeptide fused to the marker in the case of a bacterial host. Alternatively, the affinity sequence may be a hemagglutinin (HA) tag when mammalian host cells, for example COS-7 cells, are used. The HA tag corresponds to an antibody defined epitope derived from the influenza hemagglutinin protein (Wilson et al., 1984, *Cell* 37:767).

Paragraph beginning at page 30, line 10, has been amended as follows:

The nucleic acid constructs of the present invention may be in the form of RNA or in the form of DNA, such as cDNA, genomic DNA or synthetic DNA. DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. Coding sequences for mitochondrial pore components as provided herein are known in the art (see, e.g., Table 1, *infra*). For example by way of illustration and not limitation, a coding sequence that encodes an ANT or a CypD polypeptide for use according to the invention may be identical to the coding sequence known in the art for any given ANT or Cyp such as CypA or CypD, as described above and, for example, as shown for human ANT1 {~~SEQ ID NO: 1~~} (SEQ ID NO: 1), human ANT2 {~~SEQ ID NO: 2~~} (SEQ ID NO: 2) human ANT3 {~~SEQ ID NO: 3~~} (SEQ ID NO: 3), human CypA {~~SEQ ID NO: 25~~} (SEQ ID NO: 26) and human CypD {~~SEQ ID NO: 38~~} (SEQ ID NO: 39) in Figures 1, 7 and 8, or may be a different coding sequence, which, as a result of the redundancy or degeneracy of the genetic code, encodes the same ANT or Cyp polypeptide as, for example, the cDNAs of Figures 1, 7 or 8.

Paragraph beginning at page 31, line 8, has been amended as follows:

The present invention further relates to variants of the herein described polynucleotides, which may encode fragments, analogs or derivatives of native ANT and CypD polypeptides. For example, the human ANT1, ANT2 and ANT3 polypeptides and the human CypD polypeptide having the deduced amino acid sequences of Figures 2 and 8 [~~SEQ ID NOS: —~~] (SEQ ID NOS: 47-49, 27 and 40) or any ANT and CypD polypeptides may be used. The variants of the nucleic acid sequences encoding ANTs and CypDs may be naturally occurring allelic variants of the nucleic acids or non-naturally occurring variants. As is known in the art, an allelic variant is an alternate form of a nucleic acid sequence which may have at least one substitution, deletion or addition of one or more nucleotides, any of which does not substantially alter the function of the encoded ANT and CypD polypeptides. Thus, the present invention includes, for example, nucleic acids encoding the same ANT and CypD polypeptides as shown in Figures 2 and 8 [~~SEQ ID NOS: —~~] (SEQ ID NOS: 47-49, 27 and 40), as well as variants of such nucleic acids, which variants encode a fragment, derivative or analog of any of the polypeptides of Figures 2 and 8 [~~SEQ ID NOS: —~~] (SEQ ID NOS: 47-49, 27 and 40).

Paragraph beginning at page 34, line 12, has been amended as follows:

The present invention further relates to nucleic acid sequences that hybridize to ANT- or CypD-encoding polynucleotide sequences as provided herein. Preferably, such sequences display at least 70%, preferably at least 90%, and more preferably at least 95% identity to a native ANT or CypD sequence. More preferably, such nucleic acid sequences hybridize under stringent conditions to a native ANT- or CypD-encoding nucleic acid sequence. As used herein, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The nucleic acid sequences that hybridize to ANT- or CypD-encoding polynucleotides, in preferred embodiments, encode polypeptides that substantially retain a biological function or activity of an ANT or CypD polypeptide encoded by a cDNA of Figures 1 or 8 [~~SEQ ID NOS: —~~] (SEQ ID NOS: 1, 57, 3, 26 and 39).

Paragraph beginning at page 58, line 7, has been amended as follows:

In yet another apoptosis assay, induction of specific protease activity in a family of apoptosis-activated proteases known as the caspases may be measured, for example by determination of caspase-mediated cleavage of specifically recognized protein substrates. Such substrates may include, for example, poly-(ADP-ribose) polymerase (PARP) or other naturally occurring or synthetic peptides and proteins cleaved by caspases that are known in the art (*see, e.g.,* Ellerby et al., 1997 *J. Neurosci.* 17:6165). The synthetic peptide

Z-Tyr-Val-Ala-Asp-AFC [SEQ ID NO: 50] (SEQ ID NO: 50),

wherein "Z" indicates a benzoyl carbonyl moiety and AFC indicates 7-amino-4-trifluoromethylcoumarin (Kluck et al., 1997 *Science* 275:1132; Nicholson et al., 1995 *Nature* 376:37), is one such substrate. Other substrates include nuclear proteins such as U1-70 kDa and DNA-PKcs (Rosen and Casciola-Rosen, 1997 *J. Cell. Biochem.* 64:50; Cohen, 1997 *Biochem. J.* 326:1).

Paragraph beginning at page 71, line 23, has been amended as follows:

Treatment of the recombinant huANT3 protein expressed from pMK3A-huANT3 with enterokinase liberated the His-Tag/XPRESS<sup>TM</sup> epitope polypeptide from the huANT3 protein; however, the resultant huANT3 protein comprised several extraneous N-terminal amino acids (*i.e.*, Pro-Ser-Ser-Ser-Met (SEQ ID NO: 51), where "Met" indicates the amino acid encoded by the translation initiation codon of huANT3). Although the extraneous amino acids probably have little or no effect on the recombinant huANT3 protein, a derivative expression construct in which the nucleotide sequence encoding the extraneous amino acids were deleted was prepared in the following manner.

Paragraph beginning at page 77, line 16, has been amended as follows:

GST-huANT fusion proteins are further purified via glutathione-agarose beads (Sigma) essentially according to the manufacturer's instructions. In brief, a solution comprising GST-huANT fusion proteins is contacted with glutathione-agarose beads, and the beads are washed to release undesirable contaminants. Next, the [bead:GST-huANT] complexes are treated with an

appropriate enzyme, *i.e.*, one that separates the huANT polypeptide from the remainder of the fusion protein. In the case of the GST-huANT3 fusion protein described herein (*i.e.*, that encoded by pMK3C), thrombin (Sigma) cleaves the fusion protein in such a manner so as to produce two polypeptides: a first polypeptide corresponding to the GST moiety, and a second polypeptide which corresponds to human ANT3 with an additional six amino acids (*i.e.*, Gly-Ser-Pro-Gly-Ile-Leu, SEQ ID NO: 52) present at its N-terminus.

Paragraph beginning at page 77, line 27, has been amended as follows:

His-tagged huANT fusion proteins are further purified via Nickel-coated resins (such as, *e.g.*, PROBOND™ Ni<sup>2+</sup> charged agarose resin; Invitrogen) essentially according to the manufacturer's instructions. In brief, a solution comprising His-tagged huANT fusion proteins is contacted with the Nickel-coated resin, and the resin is washed to release undesirable contaminants. Next, the [resin:His-tagged huANT] complexes are treated with an appropriate enzyme, *i.e.*, one that separates the huANT polypeptide from the remainder of the fusion protein. In the case of the His-tagged huANT3 fusion proteins described herein, enterokinase (Sigma, or EKMAX™ from Invitrogen may be used) cleaves the fusion protein in such a manner so as to produce two polypeptides: a first polypeptide comprising the His-tag and XPRESS™ epitope moieties, and a second polypeptide which corresponds to human ANT3. If the expression construct used is pMK3A, the resultant purified human ANT3 protein has an additional four amino acids (*i.e.*, Pro-Ser-Ser-Ser, SEQ ID NO: 53) at its N-terminus. If pMK3B is the expression construct present in the cells from which His-tagged huANT3 is isolated, the resultant purified human ANT3 protein has the sequence of native huANT3, *i.e.*, SEQ ID NO:3.

Paragraph beginning at page 80, line 21, has been amended as follows:

The resultant GST-huANT3 expression construct was named pMK3C-GST-huANT3 (also referred to herein as pMK3C). Plasmid pMK3C has been deposited at the American Type Culture Collection (ATCC; Manassas, VA) on November 3, 1998, and given the accession number ATCC 98973. Thrombin treated recombinant huANT3 protein produced from the

pMK3C-GST-huANT3 expression construct includes several extraneous N-terminal amino acids, *i.e.*, Gly-Ser-Pro-Gly-Ile-Leu-Met, (SEQ ID NO: 54), where "Met" indicates the amino acid encoded by the translation initiation codon of huANT3. There is, however, no evidence that the extraneous six amino terminal amino acids have any effect on the resultant recombinant huANT3 protein.

In the claims:

Claims 96, 104, 107 and 108 have been amended to read as follows:

96. (Twice Amended) The method of claim 92 wherein the first isolated recombinant polypeptide comprises a cyclophilin polypeptide fused to an additional polypeptide that is selected from the group consisting of polyhistidine, polylysine, a haemagglutinin epitope tag, a DLYDDDDK {SEQ ID NO:     } (SEQ ID NO: 56) epitope tag, a DYKDDDDK {SEQ ID NO:     } (SEQ ID NO: 55) epitope tag, a Myc epitope polypeptide, a FLASH peptide, an immunoglobulin constant region polypeptide, streptavidin, a green fluorescent protein polypeptide, an aequorin polypeptide, a glutathione-S-transferase polypeptide and a *Staphylococcus aureus* protein A polypeptide.

104. (Twice Amended) The method of claim 92 wherein the second isolated recombinant polypeptide comprises a human adenine nucleotide translocator polypeptide or variant thereof that is fused to an additional polypeptide selected from the group consisting of polyhistidine, polylysine, a haemagglutinin epitope tag, a DLYDDDDK {SEQ ID NO:     } (SEQ ID NO: 56) epitope tag, a DYKDDDDK {SEQ ID NO:     } (SEQ ID NO: 55) epitope tag, a Myc epitope polypeptide, a FLASH peptide, an immunoglobulin constant region polypeptide, streptavidin, a green fluorescent protein polypeptide, an aequorin polypeptide, a glutathione-S-transferase polypeptide and a *Staphylococcus aureus* protein A polypeptide.

107. (Twice Amended) The method of claim 106 wherein the second isolated recombinant polypeptide comprises a human adenine nucleotide translocator polypeptide or variant thereof that is fused to a polypeptide selected from the group consisting of a DLYDDDDK {SEQ ID NO:     } (SEQ ID NO: 56) epitope tag and a DYKDDDDK {SEQ ID NO:     } (SEQ ID NO: 55)

epitope tag, and wherein the antibody specifically binds to at least one polypeptide selected from the group consisting of the human adenine nucleotide translocator polypeptide, the XPRESS™ epitope tag and the FLAG® epitope tag.

108. (Twice Amended) The method of claim 92 wherein the first isolated recombinant polypeptide comprises human cyclophilin D and wherein the sample which comprises the second isolated recombinant polypeptide comprises at least one submitochondrial particle isolated from a *T. ni* cell that expresses a recombinant human adenine nucleotide translocator-3 polypeptide fused to a DLYDDDDK [~~SEQ ID NO: 56~~] (SEQ ID NO: 56) epitope tag.

SJR:mls

701 Fifth Avenue, Suite 6300  
Seattle, Washington 98104-7092  
Phone: (206) 622-4900  
Fax: (206) 682-6031